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Common Variants in the 5′ Region of the Leptin Gene Are Associated with Body Mass Index in Men from the National Heart, Lung, and Blood Institute Family Heart Study

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Linkage of body mass index (BMI) to a broad region of chromosome 7q22-35 has been reported in multiple studies. We previously published a multipoint LOD score of 4.9 at D7S1804 for BMI from the National Heart, Lung, and Blood Institute Family Heart Study. Leptin (*LEP*), the human homolog of the mouse obesity (*ob*) gene, is positioned near the linkage peak and is the most prominent candidate gene in this region. Interest in *LEP* as a susceptibility gene for human obesity has led to numerous linkage and association studies, but the results of these studies are still controversial. In the present study, we employed family-based tests of association with both a quantitative measure of BMI adjusted for age and sex and a dichotomously defined obesity trait. We genotyped 29 single-nucleotide polymorphisms (SNPs) spanning 240 kb around the *LEP* gene in the 82 extended pedigrees with the strongest evidence for linkage. When the programs TRANSMIT and FBAT were used, a number of SNPs showed association in men but not women, for both the quantitative and qualitative trait definitions ($P < .05$). Five SNPs (H1328084, H1328083, H1328082, H1328081, and H1328080) positioned 2 kb beyond the previously defined promoter region showed strong association in single-marker and multiple-marker haplotype analysis. This five-marker haplotype (frequency 49% in this sample) is overtransmitted to obese offspring ($P = .00005$). All five of these SNPs are predicted to modify transcription-factor binding sites. This may indicate new functional variants in an extended promoter region of *LEP*.

Introduction

In Western societies, the prevalence of obesity has been increasing steadily in recent years. Obesity (MIM 601665) is strongly associated with increased risk for diabetes, lipid disorders, hypertension, and coronary heart disease (CHD). A genetic component in the etiology of BMI has been clearly demonstrated by epidemiological and genetic linkage studies (Borecki et al. 1998; Bouchard et al. 1998). Although many candidate genes have been proposed and numerous genetic studies performed, the resolution of the genetic factors underlying the susceptibility to obesity is far from complete (Chagnon et al. 2003).

Recently, we reported a genomewide linkage scan for BMI in the National Heart, Lung, and Blood Institute (NHLBI) Family Heart Study (FHS) (Feitosa et al. 2002). A LOD score of 4.9 was found at D7S1804 (7q32.3) for age- and sex-adjusted measures of BMI. Leptin (*LEP*) is positioned close to the center of this linkage peak and is the most prominent candidate gene in the region because of its central role in an adiposity-sensing pathway and its physiological effects on energy balance. The linkage finding at 7q22-36 has been consistently replicated by numerous studies of independent samples and supported by strong evidence of linkage in a meta-analysis of five published studies (Allison and Heo 1998; Li et al. 2003; Platte et al. 2003). The candidate region is large, extending over the 55 Mb between D7S618 and D7S3070. The positive linkage findings appear to be dispersed across three subregions. The first region is flanked by markers D7S1804 and D7S3070 at 7q32.3-q36 and is 10–15 cM downstream of *LEP*. The second region is near D7S2459/D7S523 at 7q22-31.1 and is 20 cM upstream of *LEP*. The third region is at 7q31.3, at the *LEP* gene. It is not clear whether these studies point to one or more than one gene contributing to obesity risk.

Since the identification of the *LEP* gene at 7q31.3 as the human homolog to the mouse obesity (*ob*) gene (Zhang et al. 1994), sequence variation in *LEP* has been

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studied extensively. Although mutations in the *LEP* gene are known to cause rare obesity syndromes (Montague et al. 1997*a;* Strobel et al. 1998) and have been responsible for obesity in several animal models (Zhang et al. 1994; Moon and Friedman 1997; Friedman and Halaas 1998), variation within the coding region of *LEP* is exceedingly rare in the general population (Considine et al. 1995; Maffei et al. 1996; Carlsson et al. 1997). The interest in *LEP* as a susceptibility gene for human obesity has also led to the identification of several common polymorphisms in the 5′ regulatory region—G2548A, A19G, and C1887T—that show association with lower LEP levels or obesity in several studies (Hager et al. 1998; Mammes et al. 1998, 2000; Li et al. 1999; Le Stunff et al. 2000). Nevertheless, the results from these studies are still controversial, and the relationship between *LEP* sequence variation and human body weight remains uncertain (Shigemoto et al. 1997; Lucantoni et al. 2000). In the present study, we describe the results of family-based association analysis of 29 SNPs spanning 240 kb across the *LEP* region, including the previously published SNPs and recently detected variants. Using linkage disequilibrium (LD) mapping and haplotype analysis, we sought to establish the position of the functionally significant variant(s).

Subjects and Methods

Subjects and Phenotype

The FHS is a multicenter, population-based study of factors influencing risk for CHD. A detailed description of the FHS has been published elsewhere (Family Heart Study Web site). We selected a primary genotyping panel with 548 DNAs derived from the 82 white families with the highest pedigree-specific LOD scores from our previous linkage study (Feitosa et al. 2002). The multipoint LOD score for these 82 families was 17.09 at 136.95 cM (Genetic Map Index Web site, Center for Medical Genetics).

BMI was calculated as weight (in kg) divided by the square of height (in meters). Regression models were used to adjust for sex, age, and center, as reported elsewhere (Feitosa et al. 2002). We also defined a dichotomous phenotype (denoted as "OB") based on the adjusted BMI residual (BMI-R). A residual cutoff of ≥ 0.6 was used to define affected individuals. It is notable that the residual of 0.6 corresponds approximately to a raw BMI score of 30, which is an accepted clinical designation for obesity. The dichotomous phenotype (OB) was coded as affected for those with a BMI-R ≥ 0.6, as unaffected for those with a BMI-R ≤ 0 (BMI $\leq \sim 27.5$), and as ambiguous or unknown if $0 <$ BMI-R < 0.6. Of the 82 pedigrees selected on the basis of family-specific LOD scores, 69 contained at least one individual who was classified as affected. The 69 pedigrees contained 186 persons with a BMI-R >0.6 (185 DNAs available), 196 persons with a BMI-R <0 (195 DNAs available), and 88 persons with a BMI-R between 0 and 0.6 (84 DNAs available). Most persons in the ambiguous group are founders without DNA. Nonparametric linkage (NPL) analysis was performed in the 48 families with at least two individuals classified as OB, and an NPL score of 5.16 at 136.95 cM was observed.

Genotyping

Genomic DNA was prepared from whole blood by using the Puregene system (Gentra Systems) and was purified using QIAEX II kits (Qiagen). All of the samples were quantified using the PicoGreen DNA quantification method (Ahn et al. 1996) and were diluted to 2.5 ng/μl before making 96-well PCR source plates.

Five SNPs were selected from Assays-on-Demand SNP Genotyping Products, which are validated against genomic DNAs from four different populations and each of which has a corresponding allele frequency of at least 10% in whites. Four SNPs—G2548A, C1887T, G1387A, and A19G—were selected on the basis of previous publications of association studies involving *LEP* and BMI (Mammes et al. 1998; Li et al. 1999; Le Stunff et al. 2000). An additional 20 SNPs retrieved from the human SNP database in the Celera Discovery System (Celera Discovery System Web site) were also genotyped. Twenty of these 29 SNPs are in the 22-kb *LEP* region. An additional five SNPs are from the intergenic 5′ region, two are in the *P100* gene 5′ upstream from *LEP,* and two are from the 3′ region distal to *LEP* in an unknown gene, *FLJ10377*.

SNPs were genotyped using the TaqMan technology (Holland et al. 1991; Livak et al. 1995) implemented on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). PCR was performed using TaqMan Universal Master Mix (Applied Biosystems), 5 ng DNA, 900 nM of each primer, and 200 nM of each probe in a 5-μl reaction.

Bioinformatic Analysis

Transcription-factor binding sites (TFBS) were queried for the 50-bp sequence surrounding each 5′ candidate SNP by comparing the alternative sequence of the two SNP alleles with the TRANSFAC database by use of BLAST (Wingender et al. 2000).

Statistical Analysis of Association

The genotypes were checked for Mendelian inheritance errors and Hardy-Weinberg equilibrium in an integrated database. MERLIN (Abecasis et al. 2002) was used for detection of apparent double recombinants in the data and to calculate NPL scores based on OB. Ver-

Table 1

Characteristics of Study Subjects Genotyped in the 69 Pedigrees

Characteristic	Observation in	
	Male Subjects ($N = 225$)	Female Subjects ($N = 245$)
Mean ± SD BMI:		
Total sample	29.2 ± 5.1	30.5 ± 7.6
Affected	34.8 ± 4.5 ($n = 76$)	37.4 ± 5.3 ($n = 110$)
Unaffected	25.2 ± 2.0 ($n = 97$)	23.5 ± 2.6 ($n = 99$)
Ambiguous	28.6 ± 1.2 ($n = 52$)	28.6 ± 2.2 ($n = 36$)
Mean ± SD age (in years)	51.5 ± 14.3	52.6 ± 13.1
% of sample from study center:		
NC	17.3	12.5
MN	15.0	15.8
MA	19.5	23.1
UT	48.2	48.6

sion 2.5 of the program TRANSMIT (Clayton 1999; Clayton and Jones 1999) was used to perform the transmission/disequilibrium test (TDT) of association with OB on single SNPs and multiple-marker haplotypes. *P* values for all TDT analyses performed with TRANSMIT were empirically derived from 100,000 bootstrap replicates. To protect against misleading results due to rare alleles or haplotypes, the command-line switches (i.e., flags) "-agg3" and "-c3" were used to aggregate all alleles or haplotypes with frequencies <0.03 before haplotype construction. To test for association in the presence of linkage, the switches "-1" and "-nomf" were used to select one trio from each pedigree, to minimize the dependency on family structure and to maximize the association signal relative to the linkage signal.

The software package FBAT v1.4 (Horvath et al. 2001) was used to perform the family-based association tests for the quantitative trait BMI-R in the 69 pedigrees that included at least one member who was classified as affected. The null hypothesis of linkage but no association was tested using the -e flag, which computes the test statistic through use of the empirical variance, because the sample contains multiple nuclear families in some pedigrees and multiple affected individuals in these nuclear families, and the markers are in an area of known linkage. Multiallelic tests were performed using an additive genetic model.

Since the markers are tightly linked, results for individual SNPs may be highly correlated; thus, a simple Bonferroni correction is unduly conservative, and we report the empirical *P* values as described above. We used the Benjamini-corrected false-discovery rate (FDR) to control for multiple hypothesis testing (Benjamini and Hochberg 1995). The FDR is the expected proportion of true null hypotheses rejected out of the total number of null hypotheses rejected. Multiple comparison procedures controlling the FDR are more powerful than the commonly used multiple comparison procedures based on the familywise error rate. After adjustment for multiple testing and with an FDR level of 0.05, the cutoff for significant association is $P = .01$ for analyses of "all individuals," $P = .033$ for analyses of "all males," $P = .026$ for analyses of "one trio per pedigree with affected male offspring" via a TDT, and $P = .031$ in FBAT analysis of the families with affected male offspring.

LD block structure was examined by the program Haploview (Haploview Web site, Whitehead Institute). The D' for all pairs of SNPs was calculated and the haplotype blocks estimated using the confidence-interval method (Gabriel et al. 2002). SNPs with low rare-allele frequencies may inflate estimates of D', and the use of confidence-bound estimates for D' reduces this bias. The default settings were used in these analyses, which invoke a one-sided upper 95% confidence bound of $D' > 0.98$ and a lower bound of >0.7 to define SNP pairs in strong LD. A block is identified when at least 95% of SNP pairs in a region meet these criteria for strong LD. Haplotypes were reconstructed and their frequencies estimated using an accelerated expectation-maximization (EM) algorithm similar to the partition/ligation method (Qin et al. 2002) implemented in Haploview.

Results

Characteristics of the study sample are presented in table 1. The unadjusted mean BMI in men and women was significantly different, as assessed by a *t* test ($P = .04$). This difference in mean BMI occurred in both the affected ($P = .0008$) and unaffected ($P < .0001$) groups, where women had a higher and lower mean BMI, respectively.

Table 2 shows the map locations and marker characteristics for the 29 SNPs in and around *LEP* that were genotyped. Although 4 SNPs—G2548A, A19G, C1887T, and G1387A—have been previously reported,

Table 2

Markers and Map Locations

Marker Number	Marker	Gene	SNP	Frequency[a]	Celera Location (bp)	NCBI Location (bp)	Intermarker Interval[b] (bp)	SNP Type	dbSNP rs Number
	D7S3061			NA	118093192	122844090	…	…	…
1	C1331361	*P100*	T/C	.348	122524910	127275987	4,431,897	Exon	rs322825
2	C1331345	*P100*	G/A	.190	122543069	127294146	18,159	Intron	rs6953698
3	C618722		A/G	.456	122559253	127310271	16,125	Intergenic	rs322785
4	C618685		G/C	.272	122592487	127343162	32,891	Intergenic	rs53125
5	H1331258		A/G	.351	122650302	127400977	57,815	Intergenic	rs11772985
6	H1331250		G/A	.160	122660615	127411290	10,313	Intergenic	rs6947095
7	H1328090		C/A	.389	122669402	127420076	8,786	Intergenic	rs791601
8	C1328085	*LEP*	T/G	.340	122680314	127430988	10,912	5′ flank/TFBS	rs10249476
9	H1328084	*LEP*	G/A	.411	122680501	127431175	187	5′ flank/TFBS	rs1349419
10	H1328083	*LEP*	G/A	.495	122681032	127431706	531	5′ flank/TFBS	NA
11	H1328082	*LEP*	C/A	.345	122681386	127432060	354	5′ flank/TFBS	rs12535708
12	H1328081	*LEP*	C/T	.404	122681555	127432229	169	5′ flank/TFBS	rs11770725
13	H1328080	*LEP*	C/A	.341	122681623	127432297	68	5′ flank	rs12535747
14	G2548A	*LEP*	G/A	.495	122682071	127432745	448	Promoter	rs7799039
15	H6501175	*LEP*	A/G	.446	122682636	127433310	565	Promoter	rs6467166
16	H2944325	*LEP*	G/A	.493	122682656	127433330	20	Promoter	rs12536535
17	C1887T	*LEP*	C/T	.092	122682732	127433406	76	Promoter	NA
18	G1387A	*LEP*	A/G	.446	122683231	127433905	499	Promoter	NA
19	A19G	*LEP*	G/A	.342	122684637	127435311	1,406	Exon1/5′ UTR	rs2167270
20	H1328078	*LEP*	T/C	.407	122685139	127435813	502	Intron 1	rs2278815
21	H1432616	*LEP*	G/T	.423	122690353	127441030	5,217	Intron 1	NA
22	H1432615	*LEP*	G/A	.426	122690424	127441101	71	Intron 1	NA
23	H1328076	*LEP*	A/T	.476	122691974	127442651	1,550	Intron 1	rs10244329
24	H3001671	*LEP*	A/G	.477	122693347	127444024	1,373	Intron 1	rs11763517
25	H1328074	*LEP*	G/A	.356	122694372	127445049	1,025	Intron 1	rs11760956
26	C1328073	*LEP*	G/A	.363	122694725	127445402	353	Intron 1	rs10954173
27	C3001667	*LEP*	A/G	.424	122702403	127453091	7,689	Intron 2	rs2060715
28	C1574222	*FLJ10377*	G/A	.353	122753843	127504687	51,596	3′ UTR	rs12850
29	C1328040	*FLJ10377*	C/G	.306	122768526	127519371	14,684	Intron	rs10279576
	D7S1804			NA	127031883	131246812	3,727,441	…	…

[a] Allele frequency for the second allele is shown.

[b] Marker intervals are calculated on the basis of NCBI locations (National Center for Biotechnology Information Web site).

the remaining 25 have not been studied for *LEP* association. The markers are presented according to their physical location, with D7S3061 centromeric. SNPs tested in *LEP* are intronic except for A19G, which is in the first exon, 5′ UTR region of the *LEP* gene. The rare-allele frequency for those SNPs is between 16% and 50% except for C1887T, which has a rare-allele frequency of 9%. Strong LD extends from the 5′ region to the first intron of *LEP* but does not extend to SNPs in the genes distal and proximal to *LEP* (fig. 1).

Seven LD blocks are identified across the 240-kb region (see fig. 1). We explored the haplotype diversity in this region through use of Haploview. Figure 2 depicts the values for D' between the multi-SNP blocks defined in figure 1. Haploview identified tag SNPs for parsimonious haplotypes for each block (Johnson et al. 2001; Patil et al. 2001).

In each analysis, the TDT was performed for all individuals as well as for male and female family members separately. The SNPs that remain significant after Benjamini correction are indicated with an asterisk (*). In the TDT conducted on the entire sample (the "All" column in table 3), 5 of the 20 *LEP* SNPs showed significant association with OB ($P < .05$) after Benjamini correction, but none of these markers remained significant when a "one trio only" TDT analysis was used. When sex-specific analyses were conducted, the association was seen almost exclusively among men. Overall, among all men, 16 SNPs in the "all individual" analysis ($P < .013$) and 13 in the "one trio" analysis ($P < .038$) showed significant association with OB. Although the level of significance was slightly lower in the FBAT analysis compared with the TRANSMIT results, the same pattern of association was also observed when the quantitative trait BMI-R was used. Seventeen of 20 SNPs in LEP showed significant association ($P < .031$ with Benjamini correction). In both analyses, SNPs with strong association are seen in men and are clustered between markers H1331250 and H1328076. The 117 male offspring (64 affected, 36 unaffected, and 17 ambiguous)

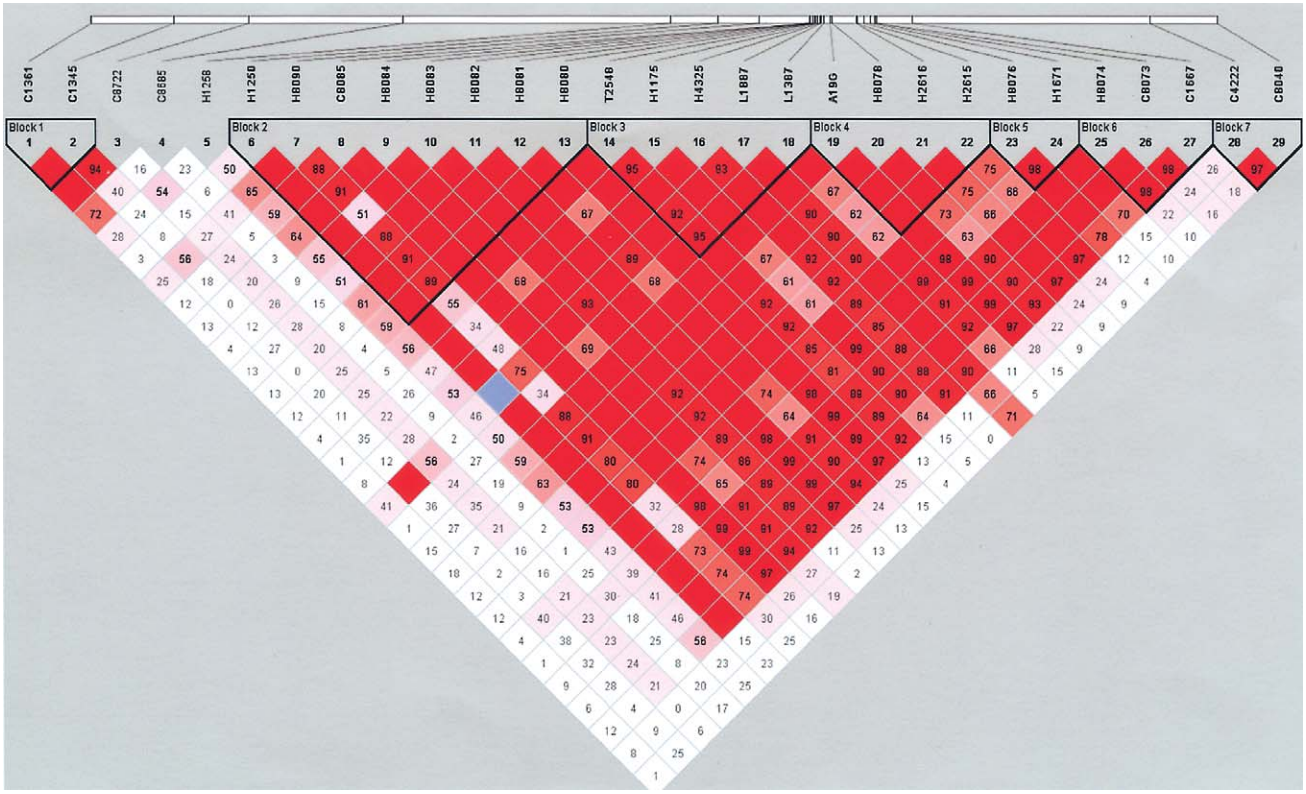


Figure 1 LD block structure around *LEP*. Haplotype block structure, as depicted by Haploview, is shown. The five-color scheme (*white* to *red*) represents the increasing strength of LD. Values for D' ($\times 100$) are shown, but those boxes with $D' = 1$ are shaded in bright red and are empty. Cells with $D' < 1$ are shades of pink or red. Blue represents $D' = 1$ but with a low confidence estimate for D'.

are very similar to the sample of all 225 male subjects described in table 1, except that they are slightly heavier (mean BMI 31.1 vs. 29.2 in the total sample) and younger (mean age 45.8 years vs. 51.5 years in the total sample).

We sought to establish the boundary of the significant association, which extended from 25 kb upstream of *LEP* to its first intron, by applying the TDT to single markers. We confirmed the previously published association with BMI at markers A19G and G2548A (Hager et al. 1998; Mammes et al. 1998, 2000; Li et al. 1999); G2548A showed a strong association with OB in both the "all individuals" ($P = .00007$) and the "one trio per pedigree" ($P = .00002$) analyses, as well as with BMI-R ($P = .003$) in the FBAT analysis. All of these associations were found solely in men. A19G showed a strong association in the TDT conducted on the entire sample ($P = .004$) but only a moderate association for the "one trio per pedigree" analysis in men ($P = .017$).

We also analyzed two-, three-, four-, and five-marker haplotypes for the 29 SNPs, and figure 3 shows the results for the three-marker haplotypes tested. The "global" P value represents the overall significance when the observed versus expected transmissions of all of the haplotypes are considered together. Although multiple LEP haplotypes showed association after correction for multiple testing, only two common haplotypes show significance globally: H1328083-H1328082-H1328081, with global $P = .006$, and H1328082-H1328081-H1328080, with global $P = .009$. The haplotype with the strongest association is a composition of allele A of H1328083, allele C of H1328082, and allele C of H1328081 (frequency 49%), overtransmitted from parents to their affected offspring ($P = .00001$).

Results of two-marker haplotype analysis resembled those for single-marker analysis (data not shown), and the four-marker and five-marker haplotype results were similar to the findings for the reported three-marker haplotypes. Among the four-marker haplotypes, two showed significant association globally: H1328084-H1328083-H1328082-H1328081 and H1328083-H1328082-H1328081-H1328080.

One common five-SNP haplotype, representing alleles G, A, C, C, and C from SNPs H1328084, H1328083, H1328082, H1328081, and H1328080, respectively, was found to be overtransmitted to affected offspring ($P = .00005$) and spans a region ~2

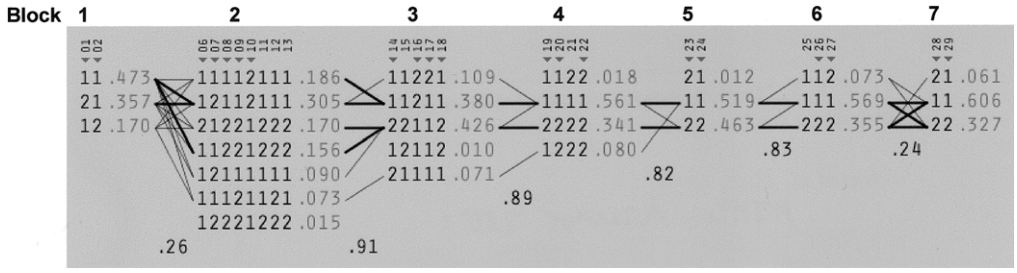


Figure 2 Haplotype structure and diversity. Haplotype blocks and their frequencies were estimated using an accelerated EM algorithm implemented in Haploview. SNP numbers corresponding to figure 1 are listed above each column of alleles, and "▼" denotes the tag SNPs that designate a parsimonious haplotype for each block. Recombination rates from one block to the next are defined by a multiallelic value of *D′*. Haplotypes in adjacent blocks are connected by a thick line if they occur together with a frequency >10% and by a thin line if they occur together with a frequency >1%. For each SNP, "1" represents the common allele, and "2" represents the rare allele.

kb up from the previously defined promoter region (Gong et al. 1996). This five-marker haplotype can be simplified to three tag SNPs—H1328081 (which is equivalent to H1328084), H1328082 (which is equivalent to C1328085 and H1328080), and H1328083—but it is not clear which combination of three is most informative. Bioinformatic analysis revealed that all five of these SNPs are predicted to modify TFBS (see fig. 4). Although G2548A shows a strong individual SNP association to OB (table 3), it is in strong LD with H1328083 ($r^2 = 0.97$), and, thus, G2548A may not add significantly to the information provided by that SNP to the common five-SNP haplotype.

Discussion

To assess the role of *LEP* in association with BMI, we performed a family-based study for both a dichotomous obesity characterization (OB) and a quantitative BMI residual measurement (BMI-R). A number of recently identified SNPs showed strong association with both of these measures in the 82 pedigrees with the strongest evidence for linkage ($P < .01$). The association signals came exclusively from men, and a sex effect of this magnitude in *LEP* has not been previously reported. The boundary of significant association extended from 25 kb upstream of *LEP* to its first intron. We identified a common risk haplotype with frequency of 49%, which is overtransmitted to obese offspring ($P = .00005$) and positioned 2 kb up from the previously defined *LEP* promoter. The five SNPs (H1328084-H1328083-H1328082-H1328081-H1328080) in this high-risk haplotype are predicted to modify TFBS, and, thus, any one of them may be functionally implicated for modifying the transcription of *LEP*. Because the SNPs are in very high LD and span a very small region (~1.2 kb), it is difficult to discern which SNP(s) is most likely implicated in *LEP* transcription. Future functional analyses using various combinations of these variants may help to confirm these findings and provide insight into the function of each variant.

In addition to the five SNPs mentioned above, we also confirmed the previously reported (Mammes et al. 1998, 2000; Li et al. 1999; Le Stunff et al. 2000) strong association between G2548A and BMI ($P = .00002$) in a single-marker TDT, but the association with this marker is not detected in any multimarker haplotype analysis. Since this SNP is neither at a conserved region among human, mouse, and rat, nor at a predicted TFBS (Li et al. 1999), its functional importance is speculative. Nevertheless, we cannot rule out the possibility that more than one functional polymorphism in *LEP* affects variation in BMI. The finding that G2548A shows strong association in single-marker analysis but does not show association in combination with other SNPs, such as those found in the five-SNP haplotype, may indicate that it is associated with an independent functional polymorphism.

The previously reported A19G SNP, in the 5′ UTR of the first exon of *LEP* (Hager et al. 1998; Li et al. 1999), shows only a modest association with OB in a stringent TDT using only one trio per pedigree ($P = .017$ in men) and a similar modest association with BMI-R in FBAT analysis. The finding that only men show *LEP* association with BMI may explain the low frequency of replication for the association of BMI with *LEP*. Furthermore, the modest effect occasionally observed for the A19G polymorphism may be attributable to strong LD between this SNP and SNPs in the haplotype ($r^2 = 0.984$ for A19G and H1328084; $r^2 = 0.681$ for H1328083), which appear to have a much stronger association with obesity.

The characteristics of promoter region for *LEP* have been reported elsewhere (Gong et al. 1996). Of note is that all five SNPs in the risk haplotype are in a 2-kb region 5′ to the reported promoter that may represent an enlarged promoter region for *LEP*. All five SNPs in this haplotype modify predicted TFBS (see fig. 4). For

Table 3

TDT of Association to OB as a Qualitative Trait in TRANSMIT, and BMI-R as a Quantitative Trait in FBAT

	TRANSMIT *P* Value[b]								
	All Individuals			One Trio per Pedigree			BMI-R (-e) FBAT *P* Value		
SNP ID[a]	All[c] (n = 470)	Female[d] (n = 423)	Male[e] (n = 315)	All[f] (n = 198)	Female[g] (n = 165)	Male[h] (n = 114)	All[c] (n = 470)	Female[d] (n = 423)	Male[e] (n = 315)
C1361	.261	.692	.220	***.018***	.261	.138	.173	.649	.093
C1345	.974	.806	.821	.976	.704	.889	.558	.614	.634
C8722	.346	.775	.255	.245	.682	***.016****	.577	.718	.100
C8685	***.027***	***.026***	.420	.493	.092	.703	.147	.104	.550
H1258	.400	.948	.093	.463	.318	.391	.426	.424	.730
H1250	***.023***	.625	***.0003****	.288	.887	***.039***	.238	.626	***.022****
H8090	.136	.259	.345	***.050***	.183	.532	.258	.648	.138
C8085	***.005****	.254	***.002****	.082	.650	***.0002****	.095	.893	***.009****
H8084	***.017***	.265	***.008****	.179	.958	***.005****	.077	.736	***.007****
H8083	.075	.947	***.001****	.618	.836	***.007****	.231	.340	***.003****
H8082	***.001****	.173	***.0004****	.107	.803	***.011****	.059	.967	***.005****
H8081	***.022***	.388	***.005****	.173	.789	***.009****	.147	.917	***.010****
H8080	***.004****	.240	***.001****	.201	.671	***.034***	.113	.708	***.008****
2548A	.114	.714	***.00007****	.714	.311	***.00002****	.246	.325	***.003****
H1175	***.036***	.665	***.0008****	.074	.663	***.0001****	.339	.317	***.008****
H4325	.132	.990	***.007****	.302	.838	***.048***	.551	.109	***.007****
1887T	.344	.121	.370	.862	.417	***.0002****	.327	.160	.361
1387A	.064	.975	***.0002****	.782	.679	***.0003****	.741	.152	***.028****
A19G	***.002****	.113	***.004****	.126	.769	***.017****	***.049***	.631	***.010****
H8078	***.006****	.307	***.001****	.133	.796	***.00002****	.073	.888	***.003****
H2616	.057	.560	***.011****	***.028***	.774	.134	.299	.586	***.014****
H2615	***.035***	.442	***.006****	***.009***	.385	***.004****	.138	.955	***.007****
H8076	.175	.879	***.005****	.150	.207	***.015****	.751	.091	***.014****
H1671	.327	.819	***.031***	.363	.576	.052	.486	.241	***.013****
H8074	.055	.418	***.031***	.240	.423	.061	.232	.777	***.034***
C8073	.072	.350	.065	.475	.855	.384	.218	.975	***.041***
C1667	.936	.304	.113	.599	.081	.107	.682	.069	.067
C4222	.072	***.021***	.613	***.028***	.249	.556	.248	.071	.970
C8040	.104	***.020***	.717	.057	.498	.988	.158	.067	.852

[a] Celera SNP IDs are abbreviated to the final four digits.

[b] *P* values are generated by simulation of 100,000 bootstrap sampling. *P* values <.05 are in boldface italic, and *P* values significant after FDR adjustment are indicated by an asterisk (*).

[c] 470 individuals in 69 pedigrees.

[d] 423 individuals in 62 pedigrees with female affected offspring.

[e] 315 individuals in 44 pedigrees with male affected offspring.

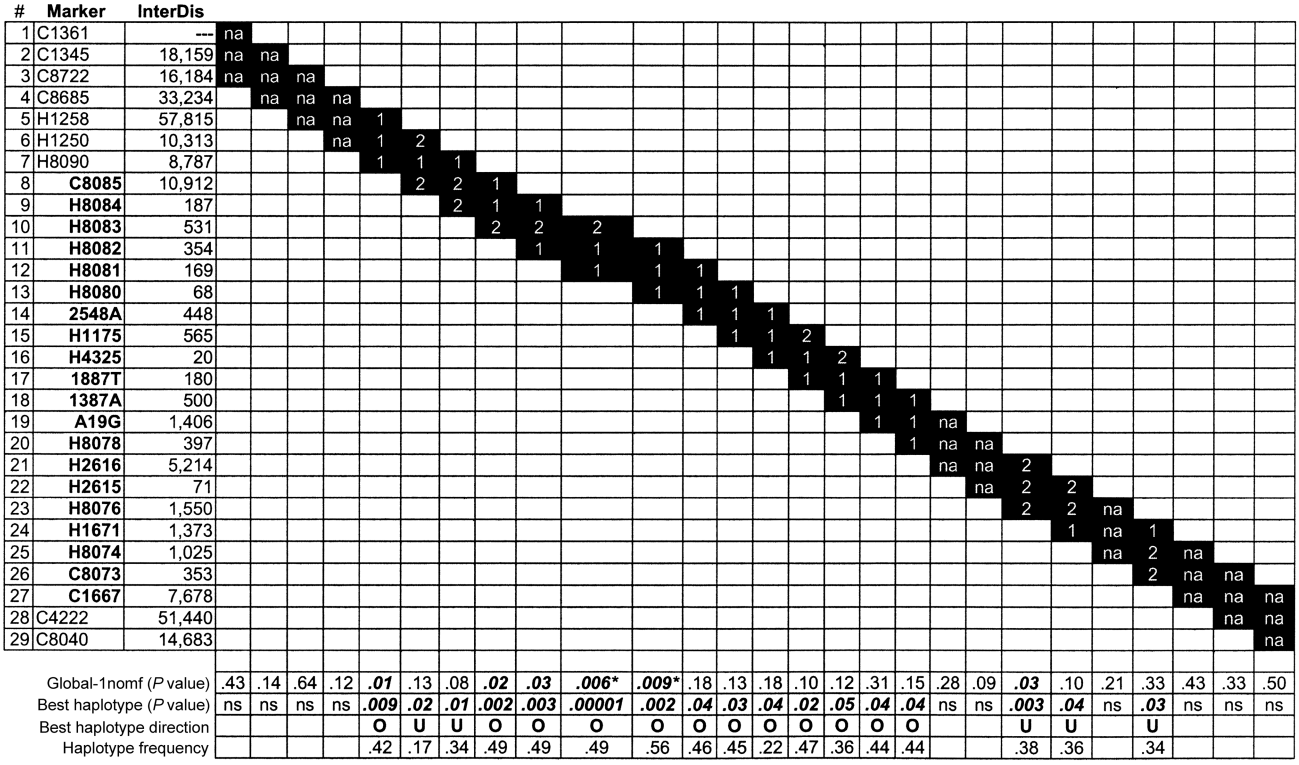
[f] 198 individuals in 66 trios with affected offspring.

[g] 165 individuals in 55 trios with affected female offspring.

[h] 114 individuals in 38 trios with affected male offspring.

example, the modification of the A allele to a C allele of SNP H1328082 results in the loss of a GATA-1 binding site. Recently, functional variation for complex phenotypes has been detected for changes other than those altering the structure of the protein encoded by the responsible gene (Toma et al. 2002). Much of the genetic component of human phenotypic diversity, including susceptibility to disease, is hypothesized to be the result of *cis*-acting influences on gene expression. If this hypothesis is correct, it implies that *cis*-acting regulatory variation may be a common phenomenon. It is also supported by some of the recent successes in the fields of diabetes (Horikawa et al. 2000), inflammatory bowel disease (Rioux et al. 2001), and schizophrenia (Stefansson et al. 2002; Straub et al. 2002), in which no obvious pathogenic coding changes have yet been identified. These observations have re-emphasized the possibility that inherited variation in gene expression may play an important role in susceptibility to complex traits such as obesity (Lander 1996; Peltonen and McKusick 2001). However, our current knowledge of regulatory elements in the human genome is far from comprehensive, and, beyond their coding sequences, most genes are not well annotated.

We genotyped the two previously reported *LEP* mutations, Met110Val and Arg105Trp (Karvonen et al. 1998; Strobel et al. 1998), and neither of them was present in our FHS samples, confirming that these mu-



#	Marker	InterDis																											
1	C1361	---	na																										
2	C1345	18,159	na	na																									
3	C8722	16,184	na	na	na																								
4	C8685	33,234		na	na	na																							
5	H1258	57,815			na	na	1																						
6	H1250	10,313				na	1	2																					
7	H8090	8,787					1	1	1																				
8	**C8085**	10,912						2	2	1																			
9	**H8084**	187							2	1	1																		
10	**H8083**	531								2	2	2																	
11	**H8082**	354									1	1	1																
12	**H8081**	169										1	1	1															
13	**H8080**	68											1	1	1														
14	**2548A**	448												1	1	1													
15	**H1175**	565													1	1	2												
16	**H4325**	20														1	1	2											
17	**1887T**	180															1	1	1										
18	**1387A**	500																1	1	1									
19	**A19G**	1,406																	1	1	na								
20	**H8078**	397																		1	na	na							
21	**H2616**	5,214																			na	na	2						
22	**H2615**	71																				na	2	2					
23	**H8076**	1,550																					2	2	na				
24	**H1671**	1,373																						1	na	1			
25	**H8074**	1,025																							na	2	na		
26	**C8073**	353																								2	na	na	
27	**C1667**	7,678																									na	na	na
28	C4222	51,440																										na	na
29	C8040	14,683																											na
Global-1nomf (*P* value)			.43	.14	.64	.12	***.01***	.13	.08	***.02***	***.03***	***.006****	***.009****	.18	.13	.18	.10	.12	.31	.15	.28	.09	***.03***	.10	.21	.33	.43	.33	.50
Best haplotype (*P* value)			ns	ns	ns	ns	***.009***	***.02***	***.01***	***.002***	***.003***	***.00001***	***.002***	***.04***	***.03***	***.04***	***.02***	***.05***	***.04***	***.04***	ns	ns	***.003***	***.04***	ns	***.03***	ns	ns	ns
Best haplotype direction							O	U	U	O	O	O	O	O	O	O	O	O	O	O			U	U		U			
Haplotype frequency							.42	.17	.34	.49	.49	.49	.56	.46	.45	.22	.47	.36	.44	.44			.38	.36		.34			

Figure 3 Haplotype analyses using one trio in each pedigree for a three-marker sliding window. Below each triad is the *P* value for that haplotype, the haplotype frequency, the direction of the transmitted haplotype (either overtransmitted ["O"] or undertransmitted ["U"]), and the global *P* value (median value of 20 runs), derived by evaluation of the transmissions of all haplotypes simultaneously. SNPs within the LEP gene are in boldface and are offset, and the approximate location within the LEP gene is shown below the haplotype frequencies. 1 = common allele; 2 = rare allele; InterDis = marker-to-marker distance (in bp); na = no haplotype resulted in $P < .05$; ns = not significant; Global-1nomf (*P* value) = global *P* value in the "one trio per pedigree" analysis. Celera SNP IDs are abbreviated to the final four digits. *P* values significant after FDR adjustment are indicated with an asterisk (*).

tations are exceedingly rare in the general population. Although the high-risk haplotype we identified is very common in FHS families showing linkage to the 7q22-35 region (49%), it is less clear how common this haplotype may be in the general population or in other genetic studies of obesity.

Sex-specific analyses show that the significant association for *LEP* was seen almost exclusively among men in this sample. This finding lends support to an unexpected sex effect between the *LEP* gene and BMI. Only two studies have included sex-stratified analyses for *LEP* and BMI or obesity (Mammes et al. 1998, 2000). These studies found that the G allele of SNP G2548A was more frequent in overweight subjects of both sexes ($P < .01$). However, only male carriers of this allele had lower LEP concentrations after adjusting for fat mass ($P = .05$). The T allele of C1887T was associated with a decrease in LEP levels in response to a restrictive diet ($P = .005$) but was not associated with BMI or LEP concentration. However, because of the small number of T-allele carriers, one cannot rule out the possibility that this decrease represents random variability. Nevertheless, these results are consistent with our finding that the common G allele of G2548A is overtransmitted in the OB offspring. A sex-specific genetic component in BMI has been reported in Norwegian twins (Harris et al. 1995) and Mexican Americans (Comuzzie et al. 1995), as well as in whites in the Framingham Study (Atwood et al. 2003). Sex-specific differences in human *LEP* mRNA expression have been implicated by previous studies (Montague et al. 1997*b*). The HERITAGE family study demonstrated that the association between the *LEP* receptor gene and adiposity was found only in white males (Chagnon et al. 2000). We may speculate that a candidate for the connection between *LEP* and sex-specific phenotypic differences is testosterone, which is down-regulated by leptin (Tena-Sempere et al. 2001). The finding that the effect of *LEP* is mainly in men, while linkage to the region is seen in both male and female subgroups in our study, suggests that there may be other important BMI-related genes in the 7q22-35 region that are worthy of additional investigation.

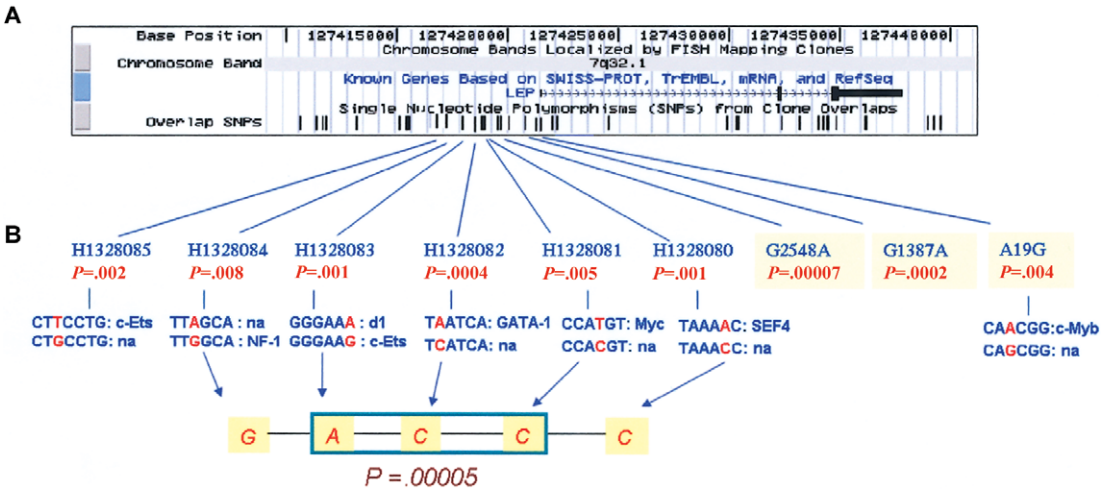


Figure 4 Genomic view of the *LEP* 5′ region, its polymorphisms, and the best five-marker haplotype. *A*, Genomic view of *LEP* mRNA and SNPs surrounding *LEP* (UCSC Genome Browser Web site). *B*, Polymorphisms at the 5′ region of *LEP*, with single-marker *P* values from TRANSMIT analysis and their respective putative TFBS, with the polymorphic site highlighted in red. No TFBS changes were found for G2548A and G1387A. na = no TFBS site identified for a specific short sequence.

In summary, the strong association of a number of common variants and haplotypes in the 5′ region of *LEP* with both a quantitative measure of BMI adjusted for age and sex and a dichotomously defined obesity trait in the present study suggest that common *LEP* sequence variants are associated with obesity in the general population. The common risk haplotype identified from this study may be clinically useful for identifying a subgroup of people who may have a *LEP* deficiency derived from altered regulatory elements and may predispose this subgroup to obesity. It will be valuable to replicate these findings in other family sets showing linkage to this region and to test for association to BMI in independent studies.

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Electronic-Database Information

The URLs for data presented herein are as follows:

Celera Discovery System, http://www.celeradiscoverysystem.com/index.cfm
dbSNP Home Page, http://www.ncbi.nlm.nih.gov/SNP/ (for SNPs [rs numbers listed in table 2])
Family Heart Study, http://www.biostat.wustl.edu/fhs/
Genetic Map Index, Center for Medical Genetics, http://research.marshfieldclinic.org/genetics/Map_Markers/maps/IndexMapFrames.html (for the Marshfield genetic map)
Haploview, Whitehead Institute, http://www.broad.mit.edu/personal/jcbarret/haploview/index.php
National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for obesity)
UCSC Genome Browser, http://genome.ucsc.edu/

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